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PARTICULATE FORMATE OXIDASE FROM *NITROBACTER AGILIS**

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SUMMARY

1. The nitrite oxidase particles obtained by sonic oscillation of *Nitrobacter agilis* cells also possessed appreciable formate oxidase activity, ranging from about 25 to 50 % of the nitrite oxidase activity depending upon the *N. agilis* strain. Both activities distributed themselves in the same pattern and proportions during differential centrifugation, and resided solely in the pellet resulting from high-speed centrifugation.

2. Difference spectra of formate-reduced particles or intact cells demonstrated the presence of cytochromes of the *c*- and *a*-types like those of the NO_2^--reduced material. Under anaerobic conditions NO_3^- or fumarate acted as an alternate electron acceptor in place of O_2 in formate oxidation. Under aerobic conditions increasing NO_3^- concentrations resulted in (a) an increased role of NO_3^- as a terminal electron acceptor compared to O_2, (b) a greater total enzymatic transfer of electrons from formate than if O_2 were the sole electron acceptor, and (c) a partial inhibition of O_2 uptake suggestive of a competition for electrons by the two acceptors. The formate oxidase system failed to catalyze consistently the transfer of electrons to either added mammalian cytochrome *c* or $Fe(CN)_6^{3-}$. The marked sensitivity of the system to certain inhibitors implicated cytochrome oxidase as an integral part of the formate oxidase. The system was also inhibited significantly by a variety of chelating agents, indicating a metal component in the formate dehydrogenase or early portion of the electron transfer sequence.

3. The stoichiometry of the formate oxidase system was shown to approach the theoretical value of 2 moles of CO_2 evolved per mole of O_2 or per 2 moles of formate consumed.

4. To a limited extent, phosphorylation occurred concomittantly with the oxidation of formate in the presence of the cell-free particulate system.

INTRODUCTION

Although *Nitrobacter agilis* possesses both conventional organic material and certain enzymes, including carboxydismutase, involved in the usual reaction required for carbon fixation[1], its catabolic metabolism of organic compounds has been presumed to be underdeveloped and is as yet poorly understood. The capacity of *N. agilis* cells

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to absorb and oxidise organic acids is limited. Formic acid oxidation by whole cells, reported first by SILVER[2] and verified by IDA AND ALEXANDER[3] and by MALAVOLTA *et al.*[4], is exceptional. MALAVOLTA *et al.*[4] showed that this activity resides mainly in the 144000 × *g* pellet (derived from cells disrupted by sonic oscillation) where the nitrite oxidase is also located[5].

The present paper describes several properties of the particulate formate oxidase system from *N. agilis*, including the similarity of its cytochrome content to that of the nitrite oxidase system as well as the demonstration of the stoichiometry of the enzymatic reaction. Phosphorylation to a limited extent appears to accompany formate oxidation.

EXPERIMENTAL PROCEDURE

Culture methods

Cells of *N. agilis* ATCC 9482 were used throughout this study except where indicated. Three other strains of this species that were studied (provided through the courtesy of Dr. Helen B. Funk) included ATCC 41423, a strain originating from Dr. M. Alexander's and one from Dr. D. Pramer's Laboratories. All strains were grown, harvested and stored as described in the preceding paper[6].

Reagents

Formate was provided as the sodium salt, "Baker Analyzed" reagent. Magnesium metal ribbon and 2,4-pentanedione for formate assays were obtained from Fisher Scientific Co. and J. T. Baker Chemical Co., respectively. Radioactive phosphorus was provided as carrier-free sodium [^{32}P]orthophosphate oral solution by E. R. Squibb and Sons. Both ADP and hexokinase were supplied by Sigma, the former as the sodium salt from equine muscle and the latter as Sigma Type III from yeast. The source and quality of all other cofactors and substances used have already been indicated in the preceding paper[6].

Preparation of cell-free extracts and localization of formate oxidase activity

Cell-free formate oxidase fractions were prepared from *N. agilis* exactly as described for the nitrite oxidase fractions[6] from the same organism. All or a major portion of the formate oxidase activity resided in the resulting 110000 or 144000 × *g* pellet which was resuspended with a Ten Broeck tissue homogenizer in 0.1 M Tris buffer (pH 7.0 or 7.5).

In the experiments concerned with intracellular localization of formate oxidase (see Table I), the centrifugation at 110000 × *g* was extended to 4 h since some activity had still not sedimented after the usual 1-h exposure to this centrifugal force.

Spectral measurements and protein determinations

Room temperature difference spectra of suspended intact cells and cell-free preparations and protein assays were determined as already described[6].

Anaerobic conditions

Anaerobic conditions, where indicated, were attained in Thunberg cuvettes by alternately evacuating and flushing 3 times with prepurified N_2 gas.

Assay procedures

Formate oxidase. Formate oxidase activity was routinely assayed by determining the rate of O_2 uptake using the Clark oxygen electrode method indicated in the preceeding paper[6]. The reaction was started by adding from a syringe 0.1 ml of enzyme preparation (approx. 0.75 mg protein), to a mixture containing 0.15 ml 0.05 M sodium formate and 0.1 M potassium phosphate buffer (pH 6.6) to give a total reaction mixture volume of 1.5 ml. The reaction was usually allowed to run for 10–15 min with the change during the first 10 min (during which time the rate was linear) taken as a measure of enzymic activity. 1 unit of formate oxidase activity is defined as the amount of enzyme which catalyses the uptake of 1.0 mμmole of O_2 per min at room temperature under the above conditions.

In reactions where NO_3^- was supplied as a competing terminal electron acceptor it was added prior to the enzyme. At the end of the reaction a sample of the reaction mixture was immediately withdrawn from the reaction chamber and assayed for NO_2^- as indicated[6].

Stoichiometry of reaction. Separate measurements of O_2 uptake and CO_2 evolution were made manometrically by the "direct method" of Warburg using constant-volume respirometers in the presence and absence, respectively, of 10 % KOH in the center well. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.6–6.8), sodium formate at 4 mM to 8 mM final concentration and about 35 mg protein of enzyme or 30–60 mg fresh weight of cells, as indicated, in a total reaction mixture volume of 3.0 ml. CO_2 was determined, after allowing for O_2 consumed, using the appropriate CO_2 solubility coefficient for pH-7.0 phosphate buffer[7]. The validity of this method for CO_2 was checked by tipping lactic acid from a side arm, at the beginning of the reaction in a control vessel, and at the end of the reaction period to release soluble and bound CO_2 from the reaction mixture.

Formate disappearance was determined by a colorimetric micromethod employing magnesium (to reduce formate to formaldehyde) and 2,4-pentanedione (acetylacetone) for color development[8]. Since NO_3^- is an alternate terminal electron acceptor and NO_2^- interferes with the colorimetric determination, particular care was taken to wash the pellet preparation as free as possible of both NO_2^- and NO_3^- before using it in formate utilization studies.

Phosphorylation. Reaction conditions for phosphorylation were similar to those used earlier to demonstrate phosphorylation coupled to the oxidation of NO_2^- as substrate[5] except that formate was employed as substrate in place of NO_2^-. The complete reaction mixture contained 15 μmoles sodium formate, 50 μmoles glucose, 0.2 μmole ADP, 15 μmoles $MgCl_2$, 5 μmoles orthophosphate containing approx. $5 \cdot 10^5$ counts/min of ^{32}P, 1.0 mg hexokinase and approx. 1.5 mg 110000 or 144000 $\times g$ pellet protein made up to a final volume of 3.0 ml (or one-half of these amounts in 1.5 ml) with 0.1 M Tris buffer (pH 7.0). Samples of reaction mixture were removed immediately after the completion of O_2 consumption measurements with the Clark oxygen electrode, processed, and assayed for organic [^{32}P]phosphate by a previously described procedure[5].

RESULTS

Distribution of formate oxidase activity

The distrubution of formate oxidase activity during the preparation of a typical pellet, is summarized in Table I. Sonicated cells showed approx. 13 % of the activity

of whole cells, with the majority of the activity residing in the subsequently prepared 10000 × *g* supernatant solution. After centrifuging the latter at 110000 × *g* for 4 h formate oxidase activity was found solely in the 110000 × *g* pellet, representing about 3 and 21 % of the total activity of the intact cells and sonicated cells, respectively, with a 3-fold increase in specific activity as compared to that of the sonicated cells. The reasons for the poor yields of enzymic activity are not clear.

TABLE I

DISTRIBUTION OF FORMATE OXIDASE ACTIVITY UPON PREPARATION OF PELLET FRACTION FROM *N. agilis* CELLS

Standard conditions of assay.

Fraction	*Total enzyme units*	*Total protein (mg)*	*Specific activity (enzyme units/mg protein)*	*% Recovery (from whole cell activity)*
Unbroken cells	96 600	—	—	—
Sonicated cells	12 520	722	17.4	12.9
10000 × *g* (10 min) pellet	1 510	606	2.5	1.6
10000 × *g* (10 min) supernatant solution	4 960	248	20.0	5.2
110000 × *g* (4 h) pellet	2 634	55	48.0	2.7
110000 × *g* (4 h) supernatant solution	0	114	0	0

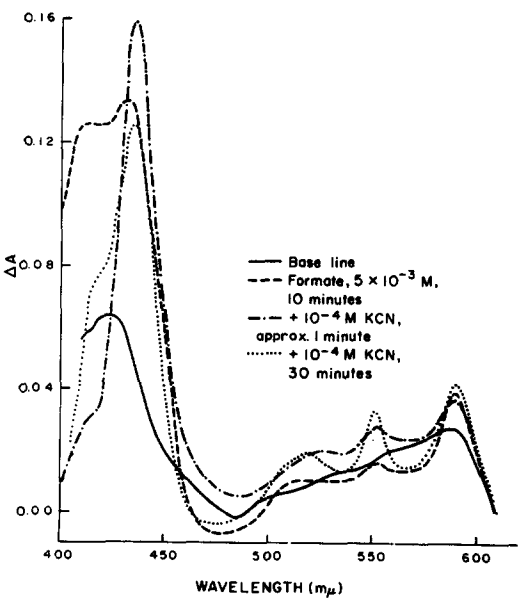

Fig. 1. Difference spectra of *N. agilis* 144000 × *g* pellet (2 mg protein) supplied 5 mM sodium formate, under steady-state conditions in 0.1 M Tris buffer (pH 7.5) and in the presence of 0.1 mM KCN (also supplied to reference cuvette) as indicated.

Cytochrome components

The addition of sodium formate to the 110000 or 144000 × *g* pellet under aerobic steady-state conditions yielded difference spectra (formate-treated *minus* untreated) demonstrating a cytochrome content (Fig. 1) similar to that obtained by NO_2^- addition reported in the preceding paper[6]. The absorption maxima at about 550 and 520 mμ with a shoulder at 415–420 mμ are representative of the α-, β-, and γ-peaks, respectively, of a *c*-type cytochrome, while the peaks at 590 and 438 mμ are of a cytochrome a_1-like component. An additional shoulder at 605 mμ indicative of cytochrome *a*, while hardly discernable here, could be demonstrated in denser pellet preparations (not shown) and in whole cells following the addition of formate. When KCN (final concn. 0.1 mM) was also provided to both cuvettes 10 min after introducing formate, the 550- and 438-mμ peaks intensified immediately whereas the 415–420-mμ shoulder lowered and narrowed; with time (30 min) the 438-mμ peak lowered, the 415–420-mμ shoulder rose somewhat, and a marked intensification of the 550-mμ peak occurred (Fig. 1). Similar spectra were obtained for the 110000 or 144000 × *g* pellet under anaerobic conditions.

Stability

The formate oxidase is heat labile, losing 43 % of its activity after 2 min at 50° and all activity after 2 min at 100°.

pH optimum

Formate oxidation catalyzed by the 110000 or 144000 × *g* pellet in 0.1 M phosphate buffer exhibited a sharp optimum at pH 6.6, with a rapid decline on either side (not shown); similar but considerably lower optima (displaying less than 20 % of the activity in phosphate buffer) were demonstrated using pyrophosphate and maleate buffers. Whole-cell oxidation of formate increased progressively as the pH was lowered from 7.2 to 6.0 using 0.1 M phosphate buffer. In 0.1 M acetate buffer an optimum was reached at pH 6.0 with a lower rate at pH 6.4 and progressively lower rates as the pH was taken below 6.0.

Affinity of enzyme for substrate

The effect of increasing formate concentrations on formate oxidase activity of the 144000 × *g* pellet is shown in Fig. 2. The K_m was estimated to be 2.8 mM. Excess formate inhibited (approx. 20 %) at 50 mM but not at 30 mM.

Electron acceptors of formate oxidase

Under anaerobic conditions at room temperature the formate oxidase system of the 110000 or 144000 × *g* pellet was able to utilize NO_3^- or fumarate as an alternate electron acceptor (in place of O_2). When either substrate was tipped into the reaction mixture from the side arm of the evacuated Thunberg tubes the reduced cytochrome peaks, that had been produced by the action of formate, rapidly disappeared. Moreover, NO_2^- production occurred following the addition of NO_3^-. Under aerobic conditions higher concentrations of NO_3^- in the presence of the 144000 × *g* pellet with formate as the electron donor were responsible for a decrease in O_2 consumption and an increased role of NO_3^- as electron acceptor compared to O_2 (Fig. 3). At 5 mM NO_3^- (the highest concentration tried) more than 90 % of the electrons transferred

from formate were contributed to NO_3^- with less than 10 % going to O_2. By contrast, with whole cells about 70 % and 30 % of the electrons were transferred to NO_3^- and O_2, respectively, possibly because of a limited permeability of whole cells to the NO_3^- provided. In both instances in the presence of O_2 and NO_3^- there was (a) a greater total transfer of electrons compared to O_2 alone by as much as 2- to 3-fold, and (b) a partial inhibition of O_2 uptake (Fig. 3).

Although reduction of endogenous cytochrome *c* by formate can be shown to occur as indicated by the difference spectra (Fig. 1), we were unable to demonstrate consistently that the 144000 or 110000 × *g* pellet catalyzed the transfer of electrons

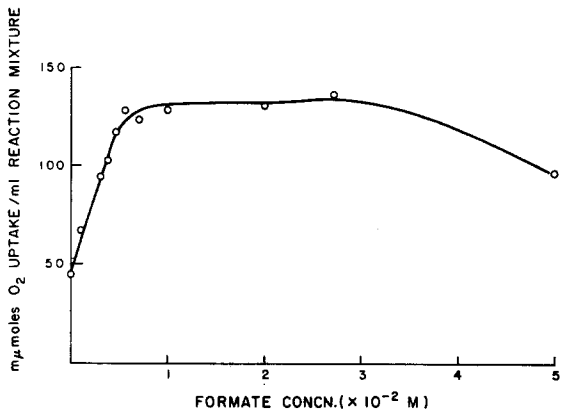

Fig. 2. Effect of sodium formate concentration on formate oxidase activity in the 144000 × *g* pellet of *N. agilis*. Standard conditions of assay.

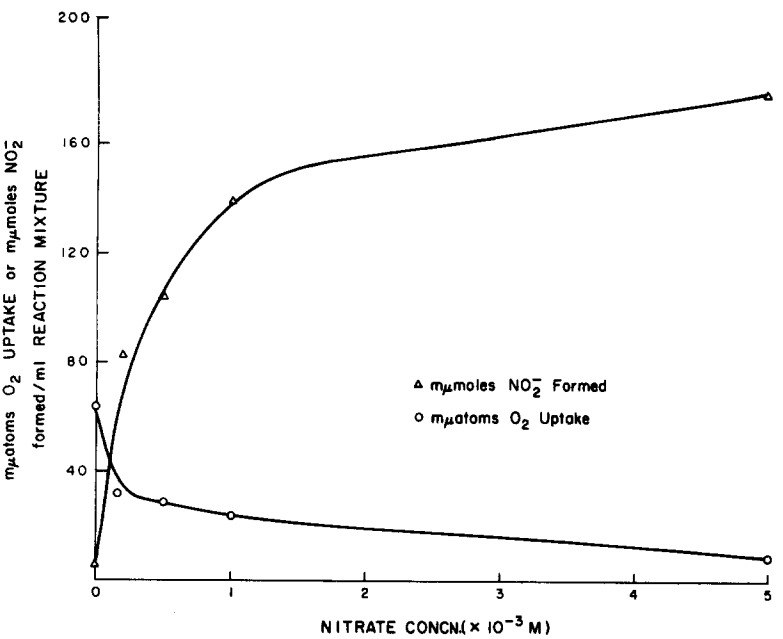

Fig. 3. Effect of increasing NO_3^- concentration on electron transport from formate to O_2 and NO_3^-, utilizing the 144000 × *g* pellet from *N. agilis*. Standard conditions of assay.

from formate to added horse-heart cytochrome *c* (0.01 %) or to 1 mM $Fe(CN)_6^{3-}$ as an alternate acceptor. Nor was it possible to show that the pellet catalyzed the reduction of 0.005 % methylene blue, 0.1 mM 2,3,6-trichloroindophenol, 1 mM phenazine methosulfate or 0.5 mM menadione by formate.

Stoichiometry

In five replicate experiments using whole cells from different batches the ratio of CO_2 evolved to O_2 consumed ranged from experimentally determined values of 1.7–2.4 with a mean of approx. 2.1 (Table II). A determination using the 144000 × *g* pellet gave a CO_2/O_2 ratio of 2.3. Two of the replicate determinations with whole cells

TABLE II

STOICHIOMETRY OF FORMATE OXIDASE FROM *N. agilis*

Whole cell Preps. 1–4 consisted of approx. 30 mg fresh weight of cells per flask and Prep. 5 approx. 60 mg. The 144000 × *g* pellet contained approx. 35 mg protein.

Preparation	*pH*	*(A) CO_2 produced (μmoles)*	*(B) O_2 consumed (μmoles)*	*(C) Formate consumed (μmoles)*	*Ratio A/B*	*Ratio A/C*
(1) Whole cells	6.8	16.5* or 15.9	7.9		2.1* or 2.0	
(2) Whole cells	6.8	6.2	2.6		2.4	
(3) Whole cells	6.8	9.2	4.2		2.2	
(4) Whole cells	6.8	6.8	4.1	8.9	1.7	2.2
(5) Whole cells	6.8	16.1	7.4	16.0	2.2	2.2
144000 × *g* pellet	6.6	4.3	1.9		2.3	

* CO_2 determined after tipping lactic acid into reaction mixture at end of reaction to release dissolved or bound CO_2 not released in control vessel at start of the reaction. See EXPERIMENTAL PROCEDURE for details.

TABLE III

EFFECT OF INHIBITORS ON FORMATE OXIDASE PELLET FRACTION OF *N. agilis*

Conditions of standard assay using 144000 × *g* pellet.

Inhibitor	*Final concn. (mM)*	*Inhibition (%)*
N_3^-	2	95
CN^-	1	94
S^{2-}	0.1	61
CO (darkness)	—	41
(after illumination)	—	0
8-Hydroxyquinoline	1	69
2,4-Dinitrophenol	0.5	100
o-Phenanthroline	5	74
Salicylaldoxime	5	53
α,α′-Dipyridyl	5	73
Diethyldithiocarbamate	5	66
NH_2OH	1	56
Amytal	2	55
Quinacrine·HCl	1	39
p-Hydroxymercuribenzoate	1	63

of the molar ratio of CO_2 evolved to formate consumed gave the same value, 2.2. In general the above results approached the anticipated theoretical ratio of 2.

Inhibitors

Formate oxidase exhibited essentially the same sensitivity to CN^-, N_3^-, 8-hydroxyquinoline, NH_2OH and diethyldithiocarbamate (Table III) as did the particulate system of MALAVOLTA *et al.*[4]. The sensitivity of formate oxidase to CN^-, N_3^- and S^{2-} as well as the light-reversible inhibition by CO are characteristic of enzyme systems containing cytochrome oxidase as an integral part of the enzymatic activity. The system is also inhibited by *o*-phenanthroline, salicylaldoxime, α,α'-dipyridyl, and 8-hydroxyquinoline, indicating a metal component, probably in the formate dehydrogenase or early portion of the electron transport chain. The inhibitory effects of 2,4-dinitrophenol and NH_2OH can probably be ascribed to their properties as metal binders. It is not clear at this time that a flavin is involved; the 40 % inhibition by 1 mM quinacrine is of itself not considered to be significant.

Of the several different metal ions added to the reaction mixture (without chelating agent) none stimulated formate oxidase activity. Ni^{2+} and Cu^{2+} inhibited 60–65 % at 1 mM, while Mg^{2+}, Zn^{2+} and Fe^{2+} inhibited about 35 % at the same concentration. Co^{2+} and MoO_4^{2-} had little or no effect.

Occurrence of formate and nitrite oxidase activities in different strains of N. agilis

A comparison of the ratio of formate oxidase to nitrite oxidase activities in whole cells (in 0.1 M phosphate buffer (pH 6.8) and initial substrate concentration (5 mM)) from small flask cultures of four different strains of *N. agilis* gave values of 0.50 for ATCC 9482, 0.35 for ATCC 14123, 0.23 for a strain originating from Dr. M. Alexander and 0.38 for a strain originating from Dr. D. Pramer.

TABLE IV

PHOSPHATE ESTERIFICATION ACCOMPANYING OXIDATION OF NADH, NO_2^- AND FORMATE BY *N. agilis*

Conditions of assay as described in EXPERIMENTAL PROCEDURE. Substrate concentration 5 mM in reaction mixture 110000 × *g* pellet.

Replicate	*Substrate*	*Counting rate (counts/min ^{32}P)*	*O_2 consumed (mμA above endogenous)*	*Phosphate esterified (mμM above endogenous)*	*P/O ratio*
1	None	166	—	—	—
	NADH	966	22	146	0.66
	NO_2^-	379	168	39	0.23
	Formate	199	44	6	0.13
2	None	228	—	—	—
	NADH	592	97	32	0.33
	NO_2^-	616	296	34	0.14
	Formate	342	44	12	0.28
3.	None	201	—	—	—
	NADH	390	91	25	0.28
	NO_2^-	337	225	33	0.28
	Formate	283	102	15	0.14

Phosphorylation

High-speed pellet preparations of *N. agilis*, which had the capacity to produce organic phosphate upon oxidation of NADH or NO_2^- (in 0.1 M Tris buffer (pH 7.0)), also carried on phosphorylation concomittant with the oxidation of formate (Table IV). However, the amount of ^{32}P fixed as organic phosphate was consistently lower for formate as compared to the other two substrates, in part because oxidation of formate is sub-optimal at this pH. Attempts to demonstrate the coupling of phosphorylation to formate oxidation using 0.02 M [^{32}P]phosphate buffer (pH 6.6) and to whole cell oxidation of formate were not successful.

DISCUSSION

The biological oxidation of formate is apparently a widespread phenomenon occurring in microorganisms, plants and animals. In *Escherichia coli* formate dehydrogenase activity[9] was originally reported to be a cytochrome-specific enzyme[10], with a requirement for iron[11,12]. More recently formate dehydrogenase activity in *E. coli* has been shown to be associated with the respiratory nitrate reductase, a particulate system which utilizes formate or NAD^+ (depending upon the conditions of growth and the *E. coli* strain employed) as an electron donor by way of cytochrome b_1 for the reduction of NO_3^- to NO_2^- (ref. 13). The relationship, if any, of this system to the formate hydrogenlyase ($HCOOH \rightarrow H_2 + CO_2$) enzyme[14] from *E. coli* is not clear. In higher plants[15–19], formate dehydrogenase shows a marked sensitivity to several metal-binding agents and a high specificity for NAD^+ as the electron acceptor ($HCOOH + NAD^+ \rightarrow NADH + CO_2 + H^+$). Formate oxidation in rat-liver and kidney extracts is also dependent on NAD^+ and is stimulated by added ATP and AMP[17].

Formate oxidation in Nitrobacter bears some resemblance to the corresponding system in *E. coli*. Both proceed by way of a cytochrome system (which, however, are not alike since cytochrome b_1 is involved in the *E. coli* sequence but apparently not in the Nitrobacter chain) and both can utilize NO_3^- as a terminal electron acceptor in place of O_2.

Of particular interest is the fact that the formate oxidase and nitrite oxidase activities of Nitrobacter share numerous, but not all, properties. First, as originally demonstrated by MALAVOLTA *et al.*[4] the formate oxidase system of Nitrobacter resides exclusively in the particulate fraction of the cell-free preparation where the nitrite oxidase also occurs. This is clearly confirmed in the present work showing a similar distribution of formate oxidase and nitrite oxidase during fractionation (*cf.* Table I with Table I of the preceding paper[6]). Secondly, a similar, if not identical, electron transfer chain is utilized at least between cytochrome *c* and O_2 for NO_2^- and formate oxidation, an observation initially made by VAN GOOL AND LAUDELOUT[20] and confirmed by the experiments reported here. The present work strongly suggests, however, that electrons from formate and NO_2^- enter the electron transport sequence either by separate dehydrogenase systems and/or at different sites, aside from the consideration of the very large difference between the oxidation potentials of the two substrates (NO_2^-/NO_3^- couple, $E_0' = +0.43$, pH 7.0; formate/CO_2 couple, $E_0' = -0.42$, pH 7.0). This is indicated by the marked inhibition of formate oxidase activity by a variety of chelating agents (Table III) which had no effect on nitrite

oxidase thus implicating a metal-containing formate dehydrogenase activity which is different from the metal-containing nitrite–cytochrome *c* reductase.

MALAVOLTA *et al.*[4] working with the 144000 × *g* pellet from Nitrobacter reported increased $^{14}CO_2$ evolution from [^{14}C]formate, with added cytochrome *c* or with added ATP. In the present study the addition of the above substances to the pellet failed to stimulate formate oxidase activity as measured by O_2 consumption. In fact ATP was inhibitory.

The present study has indicated the stoichiometry of the formate oxidase reaction to be:

$$1/2\ O_2 + H\text{–}\overset{\overset{O}{\|}}{C}\text{–}OH \rightarrow H_2O + CO_2$$

Under the conditions of our experiments little, if any CO_2 appeared to be fixed simultaneous to formate oxidation by 144000 × *g* pellet or by whole cells. Since it is the 144000 × *g* supernatant solution that presumably contains carboxydismutase and other enzymes of CO_2 fixation, the pellet alone would not be expected to fix appreciable CO_2. Under our experimental conditions whole cells also failed to fix appreciable CO_2. This is consistent both with the findings of IDA AND ALEXANDER[3] that only small quantities of CO_2 were fixed from formate by *N. agilis* whole cells, and the findings of DELWICHE AND FINSTEIN[21] that little ^{14}C from formate was fixed, compared to ^{14}C fixation from acetate or glycine.

The culture of *N. agilis* in a medium lacking NO_2^- but containing NO_3^- as nitrogen source and formate as energy and CO_2 source is theoretically feasible, if phosphorylation and the generation of reducing power in the form of NADH or a similar compound accompany formate oxidation. In the presence of NO_3^- both would be expected since there is production of NO_2^-, whose oxidation by Nitrobacter has been shown to involve both phosphorylation[5,22] and the generation of NADH[23,24]. However, attempts, to date, to culture the organism in such a medium have been unsuccessful. The obstacle has not been identified but one possibility could be the difference between the pH at which whole cell oxidation of formate occurs (optimum pH 6.0) and the pH at which cells can grow and utilize NO_2^- (optimum approx. pH 8.0).

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